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(54) Title: MODIFYING LIVING CELLS (57) Abstract A method of introducing material into living mammalian cells, or of fusing material with the cells, comprises subjecting the cells in liquid suspension in the presence of the material to ultrasonic excitation sufficient to traumatise the cells. The material introduced into the cells, or into a cell membrane, is preferably DNA or RNA or a protein.		

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MODIFYING LIVING CELLS

This invention concerns the use of ultrasound to
5 introduce material into, or fuse material with, living
mammalian cells. The technique involves traumatising
the cells, but not killing or disintegrating them.
Ultrasound involves mechanical vibration at frequencies
generally too high for the ear to detect, generally
10 from 18kHz to 20MHz.

Ultrasound has been used for diagnostic and
therapeutic purposes on living mammalian tissue. The
maximum intensity that can be applied without hazard
has been reviewed by W.D.Ulrich (IEEE Transactions on
15 Biomedical Engineering, January 1974, pages 48 to 51).

Ultrasound is widely used to decompose or
disintegrate complex chemical species, such as polymers
including DNA. Ultrasonic disintegrators for this
purpose are commercially available.

20 Scanning acoustic microscopes use low intensity
vibrations in the MHz to GHz range.

A variety of methods of introducing material into
living cells exist, including calcium phosphate
precipitation and electroporation. Electroporation
25 involves the exposure of cells to a pulsed electric
field which presumably creates pores in the plasma
membrane. It has been used to introduce DNA into both
plant and animal cells, and has been successfully
applied to a wide range of cell types which have not
30 been accessible to other methods (G.Chu et al. Nucleic
Acids Research, Volume 15 number 3 1987, pages 1311 to
1326).

Similarly, a variety of methods exists for fusing
cells, including natural methods involving viruses such
35 as Sendai or HIV, and artificial methods involving

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polyethylene glycol mediated fusion or electrofusion. The electrofusion technique comprises two main processes, dielectrophoresis, by which a close contact between cells is established, and electrical membrane breakdown which gives rise to small pores in the cell membranes resulting in fusion of two apposed membranes in close contact. (K.Ohnishi, Journal of Immunological Methods, 100 (1987) 181 to 189.

Ultrasonic forces may be used to achieve the desired close contact between cells. Ultrasound wavelengths may be used that are much smaller than the fusion chamber. This permits not only the production of pearl chains of cells (in a purely propagating wave) but also the concentration of cells at standing-wave pressure maxima. W.M.Arnold et al. (Biochemical Society Transactions, 1986, pages 246 to 249) used 1.0 MHz ultrasound (1mm wavelength) to concentrate erythrocytes or myeloma cells, for fusion by a high voltage pulse.

The present invention provides a method of introducing material into, or fusing material with, living mammalian cells, which method comprises subjecting the cells in a vessel, in the presence of the material or immediately prior to the addition of the material, to ultrasonic excitation sufficient to traumatise the cells.

Briefly cells in suspension are exposed to ultrasound frequencies in the KHz to MHz range. These frequencies can induce oscillations in the cells, or cavitation in the vicinity of the cells. The resulting stresses in the cell membranes can allow total disruption of the cell, pore formation in the cell membrane or fusion of two (or more) closely apposed cells. For the duration of this cell disruption, substances in the solution in which the

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cells are suspended may be incorporated into those cells.

The cells are subjected to ultrasonic excitation sufficient to traumatise them. This implies that the
5 cells are altered sufficient to effect entry of apposed material and, may be temporarily damaged, but not killed or disintegrated. Probably, pores are momentarily formed in the cell membrane, enabling apposed material to enter the cells or fuse with them.
10 In generating sufficient ultrasound intensity to traumatise the cells, it may be unavoidable that some of them are killed.

The nature of the mammalian cells is not critical. The cells are preferably maintained in suspension in
15 aqueous or other fluid, but may alternatively be treated while adhering to a support.

The ultrasound intensity is chosen to be sufficient to traumatise the cells but without permanently damaging or killing them. Suitable
20 intensities depend on a large number of factors, and are readily determined empirically for a particular experimental set-up. The ultrasound frequency is generally chosen in the range 18kHz to 20MHz. The time of treatment may be chosen empirically, but should
25 not be so long that an unacceptable temperature rise occurs. Treatment may be continuous or in pulses. Total treatment times of a few seconds to a few minutes are likely to be appropriate.

Materials which may be introduced into living
30 cells by this technique include, proteins, nucleic acids, oligonucleotides, DNA, lipids and lipid vesicles. Cells which take up these materials during ultrasonic excitation can survive the process and replicate subsequently. Genes introduced into cells
35 by this technique can be expressed and can be

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transmitted to daughter cells in a heritable fashion.

5 Instead of being introduced into the cell interior, materials may be incorporated by this technique into the cell membrane. Examples of materials for this purpose include lipids, hydrophobic proteins, membrane receptors, lipid vesicles and liposomes. Furthermore, ultrasonic excitation can be
10 used to induce fusion of two or more apposed similar or dissimilar cells to form a single cell.

The cells are preferably maintained in suspension in an aqueous or other liquid medium. Cell concentrations are generally in the range 10^4 to 10^8
15 cells/ml. Although the material is preferably present at the time of ultrasonic excitation, it may under some circumstances be introduced during or after the excitation while the cells remain traumatised. Preferably, the concentration of the other material
20 should also be kept at a high level. As discussed below, it is possible to use ultrasonic or other techniques to achieve localised high concentration, both of cells and of other material, in a suspension.

Various expedients, known to improve efficiency in
25 the prior art processes of electroporation and electrofusion, are also applicable in the present invention. Adjuvants may be present, including glucose/ CaCl_2 / MgCl_2 , polyethylene glycol, albumin, calmodulin, phosphatidylserine, glycerylmono-oleate,
30 cholesterol, for cell fusion. The pH, salt concentration and temperature of the suspension are all factors which may affect efficiency. For transfecting DNA into cells, the presence of carrier DNA, such as sonicated salmon sperm DNA, may increase
35 efficiency.

The cells are held in a vessel, whose size and shape and material of construction (acoustic impedance) need to be chosen in relation to the sonicating equipment being used. Small (bijou) polystyrene vials or multi-well tissue culture plates are suitable. Ultrasonic excitation may be provided by one or several ultrasonic transducers, which may be positioned above, beside or below the vessel, or may have a probe which dips into the liquid suspension in the vessel. Or the liquid suspension may be caused to flow in a controlled manner past one or more ultrasonic transducers.

Ultrasonic or other means may be provided for concentrating the cells at particular regions (e.g. standing waves) in the liquid suspension. One ultrasonic transducer may be provided to concentrate the cells, while another is operated periodically to traumatise them. Or the same transducer may be used for both purposes, with the power setting being periodically switched from a low level to a higher one. Control of the ultrasound frequency or frequency spectrum may be used to optimise the fusion of cells or uptake of material.

The following Examples illustrate the invention.

25 Example 1

Equipment

Soniprep 150 Ultrasonic Disintegrator - (MSE Ltd.,
Crawley)

Polystyrene bijoux vials
30 9cm tissue culture petri dishes

Cells

63.Ag8.653 mouse myeloma cells suspended in DMEM with
10% calf serum. DMEM is Dubbecco's modified eagles
35 medium.

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DNA

pSV2 neo/EcoR1 digest (Southern P., and Berg P., (1982) J. Mol. Appl. Genet., Vol 1, p.327). Mouse carrier DNA: high molecular weight DNA isolated from Balb/c mouse spleens and livers and partially sheared to reduce overall molecular weight.

Method

Three amplitude settings were tested for effects on cell viability.

Setting	Approximate % Survival after 10 second pulse
0	100
1	90
3	10

The cells were sonicated at the same settings in three separate bijoux (5 x 10⁶ cells in 5ml DMEM/bijou) for 10 seconds in the presence of 20ug pSV2 neo/EcoR1 digest and 50ug mouse carrier DNA.

After sonication, the contents of each bijoux were transferred to 6cm petri-dishes and cultured for two days at 37°C on standard tissue culture conditions in an atmosphere of 100% humidity, 90% air and 10% CO₂.

The cells were then transferred to large vessels thus

Setting 0	3 x 9cm petri-dishes
Setting 1	3 x 9cm petri-dishes
Setting 3	1 x 9cm petri-dishes

To each dish fresh DMEM + 10% calf serum was added to make the volume 10ml. The antibiotic G418 was then added to a concentration of 1mg/ml medium. The cells were then incubated for five days before being transferred to larger vessels. Inspection of the petri-dishes before this last transfer revealed

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numerous surviving/replicating myeloma cells in the setting 3 dish. Fewer survivors were seen in the setting 1 dish and none were seen in the setting 0 dish. Therefore the pSV2 neo plasmid DNA had entered the sonicated myeloma cells (but not the control cells) and had integrated into those cells in a heritable fashion. This plasmid conferred resistance to the antibiotic G418 on the sonicated cells. The sonicated cells therefore survived in the antibiotic whereas the control cells, which received no plasmid did not.

After a further two weeks the cells still survived in the antibiotic, indicating that the neo gene was integrated into the cells in an heritable fashion.

15

Example 2Soniffection of DNA into a Fibroblastic Cell Type Equipment

Soniprep 150 Ultrasonic Disintegrator - MSE
Linbro 24 well tissue culture plate - Flow
Laboratories

20

9cm tissue culture petri-dishes - NUNC

Cells

Psi-2 fibroblasts - a retrovirus packaging line
(Ref:- Cepko, C.L. et al., Cell 37, 1053-1062, 1984).

25

DNA

ZNR3 plasmid consisting of a cDNA encoding the human N-ras protein inserted at the Bam H1 site of the pZIP SV(X) plasmid (op. cit.)

30

Mouse carrier DNA: high molecular weight DNA isolated from Balb/c mouse spleens and livers and partially sheared to reduce overall molecular weight.

Method

Psi-2 cells were introduced into the wells of the Linbro plate in suspension at 9×10^5 cells/ml medium (DMEM - Flow Laboratories with 10% donar calf bovine serum - Flow Laboratories).

35

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As in Example 1, the tip of the exponential microprobe of the MSE Soniprep 150 was placed in the centre of the well, in this case at a depth of 1mm below the surface of the medium. Power was applied to the microprobe for 10 seconds in each case. The effects of three different "amplitudes" (as read from the scale on the MSE apparatus) were tested on cell viability:-

Setting	Viable Cells
0	8.7×10^5
1	5.4×10^5
3	1.9×10^5

For the actual experiment two wells were treated at each setting. In each well, in addition to 9×10^5 cells (initially) 10ug ZNR3 DNA and 10ug mouse-carrier DNA were included. After sonication for 10 seconds at each setting 0.2ml of cell suspension was withdrawn from each well for counting. Cell counts were as above (i.e. DNA did not alter cell viability upon sonication in this experiment).

The remaining cells were left to attach to the bottoms of the wells in which they were contained 37°C in an atmosphere of 100% humidity, 9% CO_2 /91% air.

After two days (sonication day 0 - cell splitting day 2) the cells were detached from the bottoms of the wells using a trypsin/EDTA solution (Flow Laboratories 2ml/well).

The cells were transferred to 2 x 10cm tissue culture petri dishes/well, i.e. 4 petri dishes/setting of the sonicator.

9ml fresh medium were added to each petri dish which were then returned to the 37°C incubator and

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incubated as before. The medium in this case, however, contains 1mg/ml G418. Cells which had taken up the plasmid ZNR3 were resistant to the antibiotic as the plasmid contained the same gene as that found in the pSV2 neo-plasmid of Example 1. Medium was changed on day 18. On day 20 the number of colonies of G418 resistant Psi-2 cells on the petri dishes (a colony was considered a group of greater than 16 cells) was counted.

There results were as follows:-

Setting	No. of colonies/4 petri dishes
0	0
1	207
3	0

This gives an efficiency of $10 \text{ colonies}/10^6 \text{ cells}$ surviving sonication/10ug plasmid DNA or $1/10^5 \text{ cells}/\text{ug}$.

Different cell types may need different sonicator settings and different adjuvants to achieve maximum efficiency.

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CLAIMS

1. A method of introducing material into, or fusing material with, living mammalian cells, which method comprises subjecting the cells in a vessel, in the presence of the material or immediately prior to addition of the material, to ultrasonic excitation sufficient to traumatise the cells.
2. A method as claimed in claim 1, wherein the material introduced into the cells is DNA, or RNA or protein.
3. A method as claimed in claim 2, wherein the material introduced into the cells is a gene, and the cells are subsequently caused to replicate so that the gene is expressed and transmitted to daughter cells in a heritable manner.
4. A method as claimed in claim 1, wherein the material is incorporated into the cell membrane.
5. A method as claimed in claim 1, wherein a mixture of two or more similar or dissimilar cell types is subjected to the ultrasonic excitation to induce fusion of two or more cells.
6. A method as claimed in any preceding claim, wherein the cells are maintained in suspension in a liquid.
7. A method as claimed in claim 6, wherein the cells are concentrated in suspension by means of ultrasonically induced standing waves.
8. A method as claimed in any one of claims 1 to 7, wherein, during and after ultrasonic excitation, the cells are maintained in a suitable form for reintroduction into a host mammal.

INTERNATIONAL SEARCH REPORT

International Application No **PCT/GB 88/00738**

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁴ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁴ : C 12 N 15/00; C 12 N 13/00														
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 30%; border-bottom: 1px solid black; vertical-align: top;">Classification System</td> <td style="border-bottom: 1px solid black; vertical-align: top;">Classification Symbols</td> </tr> <tr> <td style="border-bottom: 1px solid black; vertical-align: top;">IPC ⁴</td> <td style="border-bottom: 1px solid black; vertical-align: top;">C 12 N</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	IPC ⁴	C 12 N								
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III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category ¹⁰</th> <th style="width: 70%; border-bottom: 1px solid black;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%; border-bottom: 1px solid black;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="border-right: 1px solid black; vertical-align: top; padding: 5px;">X</td> <td style="border-right: 1px solid black; padding: 5px;">Chemical Abstracts, volume 72, 27 April 1970, (Columbus, Ohio, US), A.Sh. Lazaretnik et al.: "Phonophoresis in conditions of experimental tumors and leucoses", see page 187, abstract 88206s, & Fiziol. Zh. (Kiev) 1969, 15(6), 842-6</td> <td style="border-right: 1px solid black; vertical-align: top; text-align: center; padding: 5px;">1</td> </tr> <tr> <td style="border-right: 1px solid black; vertical-align: top; padding: 5px;">A</td> <td style="border-right: 1px solid black; padding: 5px;">Chemical Abstracts, volume 104, 7 July 1986, (Columbus, Ohio, US), V.I. Svidovyi et al.: "Changes in aminotransferase activity and the permeability of erythrocyte membranes in relation to ultrasound and low frequency noise", see page 442, abstract 86056y, & Gig. Sanit. 1985, (10), 73-4</td> <td style="border-right: 1px solid black; vertical-align: top; text-align: center; padding: 5px;">1</td> </tr> <tr> <td style="border-right: 1px solid black; vertical-align: top; padding: 5px;">A</td> <td style="border-right: 1px solid black; padding: 5px;">Chemical Abstracts, volume 104, 7 April 1986, (Columbus, Ohio, US), T.N. Julian: "The effects of ultrasound on drug permeation through ./.</td> <td style="border-right: 1px solid black; vertical-align: top; text-align: center; padding: 5px;"></td> </tr> </table>			Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X	Chemical Abstracts, volume 72, 27 April 1970, (Columbus, Ohio, US), A.Sh. Lazaretnik et al.: "Phonophoresis in conditions of experimental tumors and leucoses", see page 187, abstract 88206s, & Fiziol. Zh. (Kiev) 1969, 15(6), 842-6	1	A	Chemical Abstracts, volume 104, 7 July 1986, (Columbus, Ohio, US), V.I. Svidovyi et al.: "Changes in aminotransferase activity and the permeability of erythrocyte membranes in relation to ultrasound and low frequency noise", see page 442, abstract 86056y, & Gig. Sanit. 1985, (10), 73-4	1	A	Chemical Abstracts, volume 104, 7 April 1986, (Columbus, Ohio, US), T.N. Julian: "The effects of ultrasound on drug permeation through ./.	
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"G" document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; vertical-align: top;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black; vertical-align: top;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="border-bottom: 1px solid black; vertical-align: top;">29th November 1988</td> <td style="border-bottom: 1px solid black; vertical-align: top; text-align: right;">19 DEC 1988</td> </tr> <tr> <td style="border-bottom: 1px solid black; vertical-align: top;">International Searching Authority</td> <td style="border-bottom: 1px solid black; vertical-align: top;">Signature of Authorized Officer</td> </tr> <tr> <td style="border-bottom: 1px solid black; vertical-align: top; text-align: center;">EUROPEAN PATENT OFFICE</td> <td style="border-bottom: 1px solid black; vertical-align: top; text-align: right;"> P.C.G. VAN DER PUTTEN </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	29th November 1988	19 DEC 1988	International Searching Authority	Signature of Authorized Officer	EUROPEAN PATENT OFFICE	 P.C.G. VAN DER PUTTEN				
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	membrane barriers", see page 393, abstract 116006b, & Diss. Abstr. Int. B 1985 46(2), 532 --	
A	DE, A, 3505161 (GCA CORP.) 21 August 1986 --	
A	Biological Abstracts, volume 78, no. 9, 1984, (Philadelphia, PA., US), V.V. Kuklin et al.: "Using the method of protoplast fusion in the selection of Streptomyces griseus producing grinin, a strepto- thricin antibiotic", see page 7520, abstract 66698, & Antibiotiki (Mosc) 28(12): 883-889, 1983 -----	

ANNEX TO THE INTERNATIONAL SEARCH REPORT
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GB 8800738
SA 24184

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
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